ANTI-SARS MONOCLONAL ANTIBODIES

FIELD OF THE INVENTION

The present invention relates generally to the field of therapeutic or medical treatments and methods of diagnosis and detection. More specifically, the present invention relates to a plurality of anti-SARS monoclonal antibodies.

BACKGROUND OF THE INVENTION

The SARS-Coronavirus (SARS-HCoV) has been implicated as the causative agent of SARS (severe acute respiratory syndrome) in humans. This virus has caused multiple deaths in various affected countries throughout the world. The SARS coronavirus spike protein has only 30% identity at the amino acid level to the spike proteins of the previously characterised coronaviruses. Recently, the genome of SARS isolates implicated in the 2003 Toronto outbreak were sequenced in their entirety (Marco et al., 2003, Science 300: 1399-1404; Rota et al., 2003, Science 300: 1394-1399). The production of mAbs specific to this agent is critical for diagnostic development, vaccine research and studies of viral pathogenesis. Assays that detect the presence of virally encoded proteins or nucleic acids may be preferable for diagnosis of SARS infections as the development of serum antibodies is quite protracted (Li et al., 2003, N. Engl. J. Med. 349: 508-509).

Coronaviruses acre enveloped, single stranded RNA viruses that replicate in the host cell cytoplasm [Fields, B.N., Knipe, D.M., Howley, P.M., and Griffin, D.E. (2001) Fields Virology (Lippincott Williams & Wilkins, Philadelphia, ed. 4)]. The coronaviruses form a single genus of the family Coronaviridae and the virions are large (80-160 nm in diameter), pleomorphic but generally spherical particles. Virions of most coronaviruses contain three major proteins: the phosphorylated nucleocapsid protein N; a small membrane-embedded glycoprotein (M); and a large club-shaped peplomer glycoprotein (S) which appears in EM micrographs as protruding spikes 20 nm in length. The M protein is synthesized on ribosomes bound to the endoplasmic reticulum and accumulates in the Golgi apparatus. The subcellular localization of M protein to the Golgi is believed to determine the site of virus budding from the infected cell. The S protein mediates many of the biological

properties of the virus, including attachment to cell receptors, penetration, and cell-fusion, and it is the major target for virus-neutralizing antibodies (Collins et al., 1982, Virology 61:1814-1820; Talbot et al., 1984 Virology 132: 250-260; Wege and Dorrier, 1984, J. Gen. Virol. 65: 217-227; Laude et al., 1986, J. Gen. Virol. 67: 119-130; Jimenez et al., 1986, J. Virol. 60: 131-139; Godet et al., 1994, J. Virol. 68: 8008-8016). A proportion of the S glycoprotein that is not incorporated into budding virions is transported to the plasma membrane of the cell where it remains bound to the cell surface (Gerna et al., 1982, J. Gen. Virol. 60: 385-390).

Coronaviruses infect a wide range of mammalian hosts to produce a variety of disease outcomes including respiratory disease, enteritis and encephalitis. Antigenic similarities between various coronaviruses have been demonstrated to reside in the S protein and have been used to study evolution of this virus family [Brian, D.A., Hogue, B., Lapps, W., Potts, B. and Kapke, P. (1983) Proc. 4th Int. Symp. Neonatal Diarrhea (S.D. Acres, Saskatoon, Canada ed.)]. For most coronaviruses causing enteric and respiratory diseases the pathophysiological events leading to clinical symptoms are due to the acute cytocidal infection of the target cells. These infections can be limited by the local immune response resulting in the production of secretory antibodies specific for the S protein (Enjuanes et al., 1995, Dev. Biol. Stand. 84: 145-152). In contrast, many coronaviruses are maintained and spread in the population as inapparent and subclinical infections. The sequence of events leading to chronic disease is unknown but likely depends on the expression of viral genes, the functional impairment of host cells and the interaction with the host immune response.

There is a critical need to elucidate the immunologic basis for protection against SARS virus. The immunogenetics of antibody responses to protective epitopes is of particular importance and will lead to a clearer understanding of the nature of protective antibody responses to SARS. Lastly, the production of protective monoclonal antibodies may lead to the development of new recombinant therapeutic antibodies in order to provide rapid protection in SARS patients. In the present work we describe the development of murine mAbs against the SARS HCoV involved in the Toronto SARS outbreak. The mAbs were analysed for pertinent immunochemical properties and for their ability to neutralize the SARS virus in vitro.

SUMMARY OF THE INVENTION

According to a first aspect of the invention, there is provided a SARS detecting monoclonal antibody selected from the group consisting of: F26G1, F26G2, F26G4, F26G5, F26G6, F26G8, F26G12, F26G13, F26G14, F26G16, F26G17, F26G3, F26G7, F26G9, F26G10, G26G18 and F26G19.

According to a second aspect of the invention, there is provided a SARS neutralizing monoclonal antibody selected from the group consisting of F26G3, F26G7, F26G9, F26G10, F26G18 and F26G19.

According to a third aspect of the invention, there is provided a kit comprising at least one monoclonal antibody selected from the group consisting of: F26G1, F26G2, F26G4, F26G5, F26G6, F26G8, F26G12, F26G13, F26G14, F26G16, F26G17, F26G3, F26G7, F26G9, F26G10, G26G18 and F26G19.

According to a fourth aspect of the invention, there is provided a pharmaceutical composition comprising a SARS neutralizing monoclonal antibody selected from the group consisting of F26G3, F26G7, F26G9, F26G10, F26G18, F26G19 and combinations thereof and a suitable excipient.

According to a fifth aspect of the invention, there is provided a method of preparing a chimeric antibody comprising:

introducing an expression vector which comprises a nucleic acid encoding a constant region domain of a human light or heavy chain and a nucleic acid encoding a light chain variable region. selected from the group consisting of G1-light (SEQ ID No.1); G3-light (SEQ ID No.2); G6-light (SEQ ID No.3); G7-light (SEQ ID No.4); G8-light (SEQ ID No.5); G10-light (SEQ ID No.6); G15-light (SEQ ID No.7) and G18-light(SEQ ID No.8) or a heavy chain variable region selected from the group consisting of G1-heavy (SEQ ID No.9); G3-heavy (SEQ ID No.10); G6-heavy (SEQ ID No.11); G15-heavy (SEQ ID No.12) and G18-heavy (SEQ ID No.13) into a suitable host cell;

growing the host cell under conditions promoting expression of the chimeric antibody; and

recovering the chimeric antibody.

According to a sixth aspect of the invention, there is provided a method of preparing a humanized antibody comprising:

providing a nucleic acid comprising a light chain variable region selected from the group consisting of G1-light (SEQ ID No.1); G3-light (SEQ ID No.2); G6-light (SEQ ID No.3); G7-light (SEQ ID No.4); G8-light (SEQ ID No.5); G10-light (SEQ ID No.6); G15-light (SEQ ID No.7) and G18-light(SEQ ID No.8) or a heavy chain variable region selected from the group consisting of G1-heavy (SEQ ID No.9); G3-heavy (SEQ ID No.10); G6-heavy (SEQ ID No.11); G15-heavy (SEQ ID No.12) and G18-heavy (SEQ ID No.13);

modifying said nucleic acid such that at least one but fewer than about 30 of the amino acid residues of said variable region has been changed and/or deleted without disrupting antigen binding;

introducing said nucleic acid into a suitable host cell;

growing the host cell under conditions promoting expression of the humanized antibody; and

recovering the humanized antibody.

According to a seventh aspect of the invention, there is provided a pharmaceutical composition comprising a chimeric antibody as described above and a suitable carrier.

According to an eighth aspect of the invention, there is provided a pharmaceutical composition comprising a humanized antibody described above and a suitable carrier.

According to a ninth aspect of the invention, there is provided a method of preparing a vaccine comprising:

recovering from a preparation of live, attenuated or recombinant SARS virus, antigens recognized by one or more monoclonal antibodies selected from the group consisting of F26G1, F26G2, F26G4, F26G5, F26G6, F26G8, F26G12, F26G13, F26G14, F26G16, F26G17, F26G3, F26G7, F26G9, F26G10, G26G18 and F26G19.

According to a tenth aspect of the invention, there is provided a nucleic acid molecule encoding a peptide comprising a light chain variable region selected from the group consisting of G1-light (SEQ ID No.1); G3-light (SEQ ID No.2); G6-light (SEQ ID No.3); G7-light (SEQ ID No.4); G8-light (SEQ ID No.5); G10-light (SEQ ID No.6); G15-light (SEQ ID No.7) and G18-light(SEQ ID No.8) or a heavy chain variable region selected from the group consisting of G1-heavy

(SEQ ID No.9); G3-heavy (SEQ ID No.10); G6-heavy (SEQ ID No.11); G15-heavy (SEQ ID No.12) and G18-heavy (SEQ ID No.13).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: ELISA results of monoclonal antibody on whole inactivated SARS virus and BSA. Hybridoma supernatants were tested at a 1/4 dilution in PBS,0.2% BSA on pre-blocked plates, coated with 18 ng per well of inactivated virus. Positive clones were identified as having positive binding (color) in wells which were at least 4 – fold higher than the background level reactivity on BSA. Antigen Legend: Black bars - native, purified SARS-HCov ; White bars - BSA (bovine serum albumin).

Figure 2: Immunofluorescence staining of SARS HCoV-infected Vero cells with neutralizing and non-neutralizing SARS mAbs.; A. F26G6, non-neutralizing mab specific for the spike protein. B. F26G3, neutralizing mAb. C. F26G7, neutralizing mAb. D. F26G9, neutralizing mAb. E. Irrelevant mAb, F25G1. F. Irrelevant mAb F25G1 in bright field.

Figure 3. Immunohistochemical analysis of binding of mAb F26G6 to (A) SARS infected but not (B) uninfected VERO cells.

Figure 4. Western immunoblot of monoclonal antibody on whole inactivated SARS virus and infected vero cell lysates. The positive and preimmune control sera were from the corresponding immune mouse and tested at 1/2000 dilution in TBS, 0.2% BSA . Lanes marked 1 were loaded with purified virus ; 2, with infected Vero cell lysate.

Figure 5. This figure depicts Competion ELISA performed with F26G6 (anti0Spike) and F26G15 (anti-NP) mAbs on whole purified SARS virus as antigen. A dilution of each mAbs was shoosen that would produce approximately 50% maximum OD readin. Human normal and convalescent SARS-infected sera was diluted as shown at the bottom of the graph and used as a competitor for binding to the SARS antigen. A goat antimurine secondary antibody conjugated to HRP (preabsorbed with multiple species including human to remove any potantial crossreactivity) was used to detect murine mAb binding. Abrogation or reduction of the signal indicates the presence of human antibody to the same antigen/epitope in the human human serum. This indicates that the individual was exposed or

infected to the SARS corona virus. Our data also indicates that NP reactivity may be an earlier predictor of SARS infection as some sera inhibit NP bing mAb F26G15 but do not inhibit spike specific mAb F26G6. NHS=Normal human Sera tested at highest concentration 1/25 ; "S" are SARS patient convalescent sera.

Figure 6. Sequence data showing clones are unique and the id of the CDR regions that play a role in neutralization (see PDF files for improved resolution) The data shows that none of the VH or VL genes of the anti-SARS neutralizing or Western immunoblot positive mAbs are the same. This means that each hybridoma was derived from a uniques B cell and target SARS using different proteins. (ie not the same clone picked several times)

Figure 7. SARS-specific monoclonal antibodies, Heavy chains (VH) amino acid sequences.

Figure 8. SARS-specific monoclonal antibodies, Lignt chains (VL) amino acid sequences.

Figure 9. SARS-specific monoclonal antibodies, Heavy chains (VH) nucleotide sequences.

Figure 10. SARS-specific monoclonal antibodies, Lignt chains (VL) nucleotide sequences.

Figure 11. Distribution of SARS CoV in spleen, liver and lung from mice infected via IP (A), IN (B) and OR (C). Organs were collected on days 1, 3, 5 and 7 p.i. and viral RNA was detected by nested RT-PCR using a primer set against the polymerase. Number of animals that were positive by RT-PCR is shown as a percentage (n=2).

Figure 12. Number of mice that were RT-PCR positive in the spleen, lung and brain following IN inoculation of SARS CoV (A) or IP injection of neutralizing antibodies followed by IN inoculation of SARS CoV 2 hours later (B). N=5 per group and time point. Blood, liver and kidney have been omitted due to non-existent or insignificant levels of viral RNA.

Figure 13. Titres for lung samples collected from SARS CoV infected mice (IN and IN+AB groups) at various time points p.i., determined by TCID50. Values are expressed in TCID50/gram of tissue.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned hereunder are incorporated herein by reference.

DEFINITIONS

As used herein, "neutralizing antibody" refers to an antibody, for example, a monoclonal antibody, capable of disrupting a formed viral particle or inhibiting formation of a viral particle or prevention of binding to or infection of mammalian cells with a viral particle.

As used herein, "diagnostic antibody" or "detection antibody" or "detecting antibody" refers to an antibody, for example, a monoclonal antibody, capable of detecting the presence of an antigenic target within a sample. As will be appreciated by one of skill in the art, such diagnostic antibodies preferably have high specificity for their antigenic target.

As used herein, "humanized antibodies" refer to antibodies with reduced immunogenicity in humans.

As used herein, "chimeric antibodies" refer to antibodies with reduced immunogenicity in humans built by genetically linking a non-human Variable region to human constant domains.

Described herein is the isolation, identification and characterization of a plurality of anti-SARS monoclonal antibodies.

As discussed herein, some of the monoclonal antibodies have been shown to have SARS neutralizing activity, meaning that said monoclonal antibodies, humanized or chimeric versions thereof or immunoreactive fragments thereof could be used as therapeutics for treating, preventing or ameliorating symptoms associated with SARS infection in patients in need of such treatment. The patients may be for example human.

Also described herein are methods of producing anti-SARS mAbs, for example, humanized or chimeric anti-SARS mAbs. It is of note that these mAbs may be produced in a variety of systems, for example, germline cells or transgenic

plants. In these embodiments, an expression vector comprising a nucleic acid encoding an anti-SARS mAb or a humanized or chimeric version thereof or an immunoreactive fragment thereof is transformed into a suitable host and the host is grown under conditions promoting expression of the mAb which is then recovered. The mAbs may then be purified using means known in the art and used to develop pharmaceuticals, as discussed below.

As described herein, some of the monoclonal antibodies are useful for detection of SARS virus within biological samples for example, but by no means limited to, infected cells, directly on viral particle in infected cell lysates, in purified virus fractions, serum, whole blood, naso-pharengeal swabs, stool, or bronchio-alveolar lavage. As will be appreciated by one of skill in the art, individual detection monoclonal antibodies or combinations thereof may be packaged in a kit along with instructions for use, as described below.

The SARS detection monoclonal antibodies may be selected from the group consisting of: F26G1, F26G2, F26G4, F26G5, F26G6, F26G8, F26G12, F26G13, F26G14, F26G16, F26G17, F26G3, F26G7, F26G9, F26G10, G26G18 and F26G19.

The SARS neutralizing monoclonal antibodies may be selected from the group consisting of F26G3, F26G7, F26G9, F26G10, F26G18 and F26G19.

DNA and amino acid sequences for the above-referenced monoclonal antibodies may be found in Figures 7-10 and also in the attached sequence listing, wherein amino acid sequences of : F26G3-VH is SEQ ID No. 1; F26G7-VH is SEQ ID No. 2; F26G9-VH is SEQ ID No. 3; F26G10-VH is SEQ ID No. 4; F26G18-VH is SEQ ID No. 5; F26G19-VH is SEQ ID No. 6; F26G1-VH is SEQ ID No. 7; F26G6-VH is SEQ ID No. 8; F26G8-VH is SEQ ID No. 9; F26G3-VL is SEQ ID No. 10; F26G7-VL is SEQ ID No. 11; F26G9-VL is SEQ ID No. 12; F26G10-VL is SEQ ID No. 13; F26G18-VL is SEQ ID No. 14; F26G19-VL is SEQ ID No. 15; F26G1-VL is SEQ ID No. 16; F26G6-VL is SEQ ID No. 17; and F26G8-VL is SEQ ID No. 18; and wherein DNA sequences of : F26G3-VH is SEQ ID No. 19; F26G7-VH is SEQ ID No. 20; F26G9-VH is SEQ ID No. 21; F26G10-VH is SEQ ID No. 22; F26G18-VH is SEQ ID No. 23; F26G19-VH is SEQ ID No. 24; F26G1-VH is SEQ ID No. 25; F26G6-VH is SEQ ID No. 26; F26G8-VH is SEQ ID No. 27; F26G3-VL is SEQ ID No. 28; F26G7-VL is SEQ ID No. 29; F26G9-VL is

SEQ ID No. 30; F26G10-VL is SEQ ID No. 31; F26G18-VL is SEQ ID No. 32; F26G19-VL is SEQ ID No. 33; F26G1-VL is SEQ ID No. 34; F26G6-VL is SEQ ID No. 35; and F26G8-VL is SEQ ID No. 36.

As will be appreciated by one of skill in the art, the monoclonal antibodies may be used individually or in any combination thereof.

As will be appreciated by one of skill in the art, detection antibodies must show high specificity and avidity for their antigenic target. As such, showing that a monoclonal antibody reacts with the antigenic target derived from a highly purified or in vitro prepared sample does not guarantee that the antibody has sufficient specificity for use with biological sample. That is, the monoclonal antibody must have sufficient specificity that it will not produce false positives or react with antigens from related, non-SARS coronaviridae.

Examples of suitable tests for determining utility as a diagnostic or as a neutralizing mAb include but are by no means limited to negative neutralization and/or negative detection of a non-SARS coronavirus, C-ELISA data showing competition of binding with the mouse mAbs that is being detected thereby showing that the mAbs can be used to show that an immune response to SARS has occurred in patient/animal sera, meaning that they were exposed/infected (abrogation of binding by human antibodies). Alternatively, biological material such as blood, mucus or stool with could be spiked or enriched with the virus and the monoclonal antibodies used to detect added virus in the sample, which would in turn determine limits of detection as well as other parameters of the monoclonal antibodies. Biological samples from experimentally infected animals could also be used to determine the utility of the mAbs at different stages of the infection cycle. Yet another method, although less desirable, would be testing of the patient material from the outbreak as this is scarce and hence valuable material.

In use, at least one of the detection antibodies is mixed with a biological sample under suitable conditions to promote binding of the at least one detection antibody with the antigenic target if the antigenic target is present in the biological sample. Binding of the detection antibody to an antigenic target within the sample is then detected using means known in the art, for example, by use of a labelled secondary antibody or other means discussed herein and/or known in

the art.

As will be apparent to one of skill in the art, a combination of detection antibodies may be used. Furthermore, at least one of the detection antibodies or combinations thereof may be packaged in a kit for detecting SARS virus in biological samples. The kit may include instructions and additional reagents, for example, secondary antibodies, buffers, detection reagents and the like. Antibodies of the kit could be used for example in a capture ELISA wherein one or more mAb is coated onto a surface to catch and present SARS antigen from biological samples, then another prelabelled mAb is added to detect the presence of the antigen; as a control for indirect ELISA wherein a surface is coated with SARS antigen and the presence of antibody binding to the antigen is detected; for immunoflourescence; or for competition ELISA wherein SARS antigen is coated on a surface, and the ability of human or other infected/exposed animal serum antibody to prevent binding of one or more of the mAbs to the SARS antigen is measured.

The neutralizing antibodies were previously shown to react with a conformational epitope of the native virus which is abrogated upon denaturation of the virus. However, as will be appreciated by one of skill in the art, this does not guarantee that the neutralizing antibodies will be effective in either preventing virus formation or disrupting intact virus particles *in vivo*, that is, that the neutralizing antibodies will have therapeutic activity.

For example Maruyama et al demonstrated in vitro neutralization using monoclonal antibodies to Ebola virus and Parren et al confirmed this observation in guinea pigs; however in non-human primates there was no protection afforded by the monoclonal antibody. Furthermore, Jones et al. conducted extensive studies to identify which monoclonal antibodies were protective against infection with the bacteria Burkholderia pseudomalei. Whilst the in vitro neutralization is an excellent screening assay, the definitive test for neutralization is the in vivo protection assay. (Maruyama et al., J Virol. 1999; 73(7):6024-30; Parren et al., J Virol. 2002; 76(12):6408-12; Jones et al., J Med Microbiol. 2002;51(12):1055-62).

It has also been shown in HIV that in vitro neutralizing antibodies may not protect against primary isolate in vivo (Poignard et al., J Virol. 2003 Jan;77(1):353-65). In addition, mAbs that recognize the same region (epitope) but

in different ways may have different neutralization properties, that is, one may neutralize while another may not, clearly indicating that neutralization is entirely empirical and needs to be tested. (Parren et al., J Virol. 1998 Dec;72(12):10270-4).

In another embodiment of the invention, a nucleic acid sequence encoding the neutralizing antibody as described above is subjected to humanization techniques or converted into a chimeric human molecule for generating a variant neutralizing antibody which has reduced immunogenicity in humans. Humanization techniques are well known in the art – see for example US Patent 6,309,636 and US Patent 6,407,213. Chimerics are also well known, see for example US Patent 6,461,824, US Patent 6,204,023, US Patent 6,020,153 and US Patent 6,120,767.

In one embodiment of the invention, chimeric antibodies are prepared by preparing an expression vector which comprises a nucleic acid encoding a constant region domain of a human light or heavy chain genetically linked to a nucleic acid encoding a light chain variable region selected from the group consisting of G1-light (SEQ ID No.1); G3-light (SEQ ID No.2); G6-light (SEQ ID No.3); G7-light (SEQ ID No.4); G8-light (SEQ ID No.5); G10-light (SEQ ID No.6); G15-light (SEQ ID No.7) and G18-light(SEQ ID No.8) or a heavy chain variable region selected from the group consisting of G1-heavy (SEQ ID No.9); G3-heavy (SEQ ID No.10); G6-heavy (SEQ ID No.11); G15-heavy (SEQ ID No.12) and G18-heavy (SEQ ID No.13). It is of note that all of these sequences are shown in Figures 7-10.

In another embodiment of the invention, there are provided recombinant anti-SARS antibodies comprising at least one modified variable region, said region selected from the group consisting of G1-light (SEQ ID No.1); G3-light (SEQ ID No.2); G6-light (SEQ ID No.3); G7-light (SEQ ID No.4); G8-light (SEQ ID No.5); G10-light (SEQ ID No.6); G15-light (SEQ ID No.7); G18-light(SEQ ID No.8); G1-heavy (SEQ ID No.9); G3-heavy (SEQ ID No.10); G6-heavy (SEQ ID No.11); G15-heavy (SEQ ID No.12) and G18-heavy (SEQ ID No.13) in which at least one but fewer than about 30 of the amino acid residues of said variable region has been changed or deleted without disrupting antigen binding. It is of note that all of these sequences are shown in Figures 7-10.

In yet other embodiments, immunoreactive fragments of any of the above-described monoclonal antibodies, chimeric antibodies or humanized antibodies are prepared using means known in the art, for example, by preparing nested deletions using enzymatic degradation or convenient restriction enzymes.

It is of note that in all embodiments describing preparation of humanized antibodies, chimeric antibodies or immunoreactive fragments of monoclonal antibodies, these antibodies are screened to ensure that antigen binding has not been disrupted. This may be accomplished by any of a variety of means known in the art, but one convenient method would involve use of a phage display library.

The nucleotide sequence encoding the variable regions of the light and heavy chains of antigen specific hybridomas represent the specificity of the anitbody. Specifically the most important regions are the CDRs (of the light and heavy chains): L1, L2, L3 and H1 H2 H3 respectively. It will be apparent to one of skill in the art that the most importance CDR domains are those that are most variable in nature and thus are recruited most specifically by a given antigen like SARS. These are L1 and H3. Residues in H3 and other CDR comprise the paratope which interacts with the epitope on the pathogen. Amino acid residues in H3 have have been shown to directly interact/bind to residues of the epitope in crystal structure determinations. (Bossart-Whitaker et al., J Mol Biol. 1995 Nov 3;253(4):559-75; Chavali et al.,.Structure (Camb). 2003 Jul;11(7):875-85; Afonin et al., Protein Sci. 2001 Aug;10(8):1514-21; Karpusas et al., J Mol Biol. 2003 Apr 11;327(5):1031-41; Krykbaev et al., J Biol Chem. 2001 Mar 16;276(11):8149-58. Epub 2000 Nov 01; Beiboer et al., J Mol Biol. 2000 Feb 25;296(3):833-49; Haruyama et al., Biol Pharm Bull. 2002 Dec;25(12):1537-45).

It is of note that as discussed herein, the above-described neutralizing antibody or humanized variant thereof may be formulated into a pharmaceutical treatment for providing ~~passive~~ immunity for individuals suspected of or at risk of SARS infection comprising a therapeutically effective amount of said antibody. The pharmaceutical preparation may include a suitable excipient or carrier. See, for example, Remington: The Science and Practice of Pharmacy, 1995, Gennaro ed. As will be apparent to one knowledgeable in the art, the total dosage will vary according to the weight, health and circumstances of the

individual as well as the efficacy of the antibody.

In another embodiment of the invention, a vaccine is prepared by recovering from a preparation of live, attenuated or recombinant SARS virus, antigens recognized by one or more monoclonal antibodies selected from the group consisting of F26G1, F26G2, F26G4, F26G5, F26G6, F26G8, F26G12, F26G13, F26G14, F26G16, F26G17, F26G3, F26G7, F26G9, F26G10, G26G18 and F26G19.

The invention will now be described according to examples; however, the invention is not limited to or by the examples.

Immunization and Virus Antigen Preparation:

For immunizations 5-6 week old female BALB/C mice were used (Charles River). The mice were injected subcutaneously (S.C.) with 50-ug of beta-propiolactone-inactivated SARS-coronavirus (Tor-3 strain) with an equal part of Complete Freund's Adjuvant [CFA, H37 Ra; Difco]), on day 1. The virus had been expanded after plaque purification in Vero-6 cell monolayers and partially purified through a sucrose cushion. Highly purified SARS-coronavirus (Tor-3) was prepared the same as above except that the viral particles were further purified using gradient centrifugation. (Highly purified SARS CoV was prepared as follows briefly, 500 ml of supernatant from SARS CoV infected Vero-6 cells was concentrated first on top of a cushion of iodixanol in a SW32 rotor (Beckman). Subsequently, the virus was mixed to form a suspension of 20% iodixanol and centrifuged in a NVT 90 rotor (Beckman) for 3.5 hours at 400,000g. Fractions were collected from the bottom of the self-generated gradient, tested by Western immunoblot with convalescent patient serum, and the SARS CoV positive fractions were pooled and dialysed against PBS. The dialysed virus preparation was further concentrated by ultracentifugation for 1.5 hours at 150,000g. On day 30 the mice received 50 µg of purified SARS virus antigen S.C. in Incomplete Freund's Adjuvant (IFA) in a total volume of 100 µl. On days 48 and 63, the mice received 5 µg of the same antigen in a total volume of 100 µl S.C. with IFA. Mice received a final booster injection with 5 µg of purified SARS urus in 200 µl PBS to the intra-peritoneal cavity 3 days prior to hybridoma fusion. Mice were euthanised by anaesthesia overdose and exsanguinated by cardiac puncture. The spleens were subsequently excised under aseptic conditions.

Preparation of Infected Cell lysate

Infected Vero cells were scraped off of 162 cm^2 tissue culture flasks (Corning) and centrifuged for clarification. A borate saline mixture (0.05 M boric acid, 0.12 M, NaCl, 0.024 M NaOH) was used to wash the cell pellet twice and the pellet was resuspended in 2 ml borate saline + 1 % triton x-100 for each T162 flask. The pellet was kept at 4°C using a water bath and sonicated for ten minutes at 50% power. The debris was pelleted via centrifugation at 10,000 X g for ten minutes and the supernatant collected and stored at -20°C in aliquots for later use.

Generation of mAbs:

Immunization of mice, removal of spleens, preparation of spleen and myeloma cells, and the fusion for hybridoma production were performed according to NCFAD standard operating procedures under IS017025. Ampules of the myeloma cell line P3X63Ag8.653 (ATCC) were thawed one week prior to fusion and grown in BD Cell Mab Quantum yield media in the presence of 8-Azaguanine (Sigma). Cells were in log-phase growth at the time of fusion. Hybridoma fusion was performed essentially as originally described (Kohler and Milstein, 1975, Nature 256: 495-497) with the following modifications. Briefly, spleens were harvested 3 days after a final boost and the splenocytes were prepared by splenic perfusion as follows. A 10 cc syringe with a 21 gauge sterile disposable needle was used to perforate the spleens under aseptic conditions. The spleen cells were perfused out of the spleen with injections of serum free BD cell Mab Quantum Yield medium (BD-Pharmingen). Two identically immunized mouse spleens were used to produce these hybridoma clones. The fusion was performed using the P3X63Ag8.653 myeloma line in log phase growth. The PEG1500, 1 ml, (Roche) was added drop-wise over one minute while gently tapping the tube containing the thoroughly washed myeloma-splenocyte pellet. The PEG was slowly diluted out over three minutes with serum free BD-Cell Mab Quantum Yield media (BD-Pharmingen). The cells were resuspended and mixed into 90 ml of Clonacell Medium D (HAT) media (Stemcell, Vancouver) containing 5 ml HCF, and plated out according to the manufacturers instructions. The plates were kept in a 37°C incubator under 5% CO_2 overlay for about 10-18 days in humidified chambers.

Visible colonies were picked from the plates after about 2 weeks growth and placed into 96 well plates containing 150-200 µl of complete hybridoma medium (BD-Quantum Yield) with 1 X HT (Sigma), 4% Hybridoma cloning factor (Igen) and 10% FBS (Wisent). Supernatants were screened 4 days later via ELISA on purified virus as antigen. Isotyping was performed using a commercial dipstick test (Roche) according to the manufacturer's instructions. Hybridoma culture supernatants were concentrated 5-10 fold using stirred cell nitrogen concentrators (Amicon) with a 30 kilodalton cutoff membrane (Millipore).

Immunoassays

Enzyme linked immunosorbent assay

Tissue culture supernatants were assayed for binding to purified SARS coronavirus in an ELISA assay when the cultured cells were confluent in the culture plates. The Costar 3690 96-well ½ well ELISA plates (Corning) were coated with either Bovine serum albumin or purified SARS-coronavirus (18 - 37 ng/well) in PBS overnight at 4°C and then blocked with 0.4% BSA in PBS, for 2 hours at 37°C. The supernatant (30 µl/well) was incubated neat for 1 hour at 37°C. The ELISA plates were washed ten times with dH20 and patted dry on a paper towel. A pan-goat anti-mouse IgG-HRP antibody (Southern Biotechnology Associates) was diluted to 1:2000 in 0.2% BSA in PBS, applied to the ELISA plates for 45 minutes at 37^{0}C, and then washed as described above. Positive binding was detected with commercial ABTS used according to the manufacturers instructions (Roche). The OD was read at 405nm at 15 and 60 minute intervals after addition of the developing reagent. Mouse immune and preimmune sera was diluted to 1:2000 in 1.5 ml Eppendorf™ tubes (Falcon) in 2%-BSA PBS for use as controls.

Western Immunoblots

Whole virions or SARS-infected Vero cells at a total protein concentration of 1 ug per lane were loaded in criterion pre-cast gels (BIO-RAD) and electrophoresed at 200 V for 30 minutes. The proteins were transferred to Immobilon nylon membranes (Millipore) for 2 hours at room temperature at 100 volts, or at 27 volts overnight at 4°C. Blots were blocked in 3% BSA-TBS, rinsed

three times with TBS, and reacted with monoclonal antibody overnight at 4°C. The antibody supernatants were reacted neat and concentrated supernatants were diluted 1:50 in 0.2% BSA-PBS. Blots were washed three times with TBS-tween-20 (0.05%) for five minutes before being incubated with secondary antibody (same as above) at 1:1000 in TBS, 0.2% BSA for 1 hour. The blots were washed as above and developed using DAB (Pierce) insoluble substrate.

Immunofluorescence Staining of Vero cells infected with SARS-coronavirus

Monolayers of SARS-infected Vero cells were stained as follows. Glass slides were coated with infected Vero cell monolayers and fixed with acetone. The slides were irradiated with 20 kilogreys from a cobalt gamma irradiator, removed from biocontainment, and then stored at -80°C. Dilutions of antibodies and test sera were made initially in 96 well plates (Falcon). Samples were allowed to incubate for 45 minutes in a 37°C incubator, and were washed with distilled water. Fluorescein labelled secondary antibodies (Sigma) diluted in PBS were added to the slides and incubated for 45 minutes at 37°C, washed as above, and air dried. Slides were coated with mounting medium and stored at 4°C until examined.

Virus Neutralization

Plaque reduction virus neutralization assay (NML)

A standard plaque reduction neutralization test was performed as previously described (Godet et al., 1994, J. Virol. 68: 8008-8016). Briefly, mixtures of pre-titred (100 PFUs) SARS coronavirus and serial 2-fold dilutions of hybridoma supernatant were incubated at 37°C for 1 hr and added to six well plates containing Vero cell monolayers. After a 37°C incubation for 1 hr, a nutrient-agar overlay was added and the plates placed in a CO_2 incubator for approximately 3 days. A second overlay was then added which contained neutral red as a vital stain. Plates were then checked periodically over the next few days for plaque formation. The highest dilution tested that produced a plaque reduction of at least 90% was defined as the titration end point.

Cytopathic effect (CPE) reduction virus neutralization assay(NCFAD)

The ELISA positive monoclonal antibodies were screened for cross-

neutralization with other coronaviruses using microtiter format CPE reduction assay: concentrated monoclonal antibodies (hybridoma supernatants) were diluted 1:20 in cell culture medium and incubated with 100 TCID50 of either SARS HCoV (Tor-3), or transmissible gastroenteritis virus (TGEV, Diamond strain; kindly provided by Dr. Susy Carman, LSD, University of Guelph) for 1 hr at 37°C. The virus-antibody mix was then transferred onto cell monolayers in 96-well plates (Costar, Corning, NY). Vero V-76 cells were used for the SARS WCoV, ST cells for the TGEV. The plates were incubated until CPE developed in virus back titration controls.

Development of mAbs to the SARS-virus

We developed a panel of mAbs to the SARS HCoV. ELISA screening on purified SARS coronavirus identified a panel of 17 IgG/K type mAbs (Figure 1a, table 1). The general binding reactivity of these mAbs is decreased on heat denatured purified virus preparations indicating destruction of epitopes. There is a similar decrease in binding by many of these mAbs when tested on SARS-HCoV infected vero cell lysates as antigen. Heat denaturation had little effect on the binding of mAb F26G16 which also maintains a high OD on infected lysates. This mAb does however show higher background on the irrelavant antigen bovine serum albumin (BSA) (figure 1a) and has inconsistent reactivity in immunoblots with heat denatured viral lysate (table 1). Immunoblot methods are less sensitive than ELISA especially when using the lower quality infected cell lysate as antigen. Unfortunately preparation of highly purified viral antigen requires enormous efforts under containment which emphasizes the need for a quality recombinant antigen assay.

Western immunoblot analysis identified mAbs to the SARS spike protein. A total of five mAbs react with the SARS-spike protein in Western immunoblots, using the whole purified virus or virus infected cell lysate (Figure 1b). The antigen identity of the remaining 11 Western immunoblot negative mAbs could not be determined which suggests that these mAbs target conformational epitopes that are destroyed in the Western blot sample preparation and membrane transfer process. These data led us to test for biological activity in virus neutralization assays.

Immunochemical and Biological Characterization of binding

Neutralizing antibodies to the SARS virus recognize epitopes via interaction with both conformational and linear epitopes. We identified mAbs that neutralize in vitro cell culture infectivity of the SARS-virus. Concentrated culture supernatants from four of the eleven Western immunoblot negative (conformational) mAbs were significantly neutralizing compared to irrelevant isotype-matched concentrated mAbs to other antigens (Table 1). SARS virus infectivity was neutralized with mAbs F26G3, G7, G9, G10, G18 and G19. No cross-neutralization was observed for the animal coronavirus TGEV. The remaining mAbs in our panel showed no decrease in virus growth. This result reveals that we have developed mAbs specific for epitopes on the SARS coronavirus.

Immunoblot analysis reveals a spectrum of conformational requirements for binding. We examined the effects of different denaturing treatments on binding activity of a subset of neutralizing and some non-neutralizing mAbs using immunodot blot assays on infected lysates compared to uninfected lysates. A series of conditions were tested including exposure to heat, detergent, a reducing agent, and combinations thereof. The Immunodotblot reactivities of this panel of mAbs reveals important immunochemical requirements for their respective epitopes, and are summarized in table 1. In general the conformational requirements of the neutralizing antibodies are higher than the non-neutralizing and they are less tolerant of denaturation of the epitopes. None of the mAbs react with mock-infected lysates as assayed in Immunodotblots. This suggests that the majority of the neutralizing mAbs likely target surface exposed protein epitopes on the native viral particle, which has been identified as spike protein via Western analysis for mAbs F26G18 and F26G19. This is consistent with binding data observed in ELISA on heat denatured virus infected lysate compared to native infected lysate. In this case, regardless of Western reactivity, the non-neutralizing clones retain more ability to bind to heat denatured antigens compared to neutralizing mAbs (lower mean percent reduction in OD per group $p<0.001$, students T test). There are exceptions, however, in that it is difficult to use traditional classifications to describe the binding properties of these mAbs as being conformational or linear according to biological activity. Interestingly, clone

F26G18 binds to spike protein in Western blot and neutralizes the SARS virus and thus the binding of F26G6 cannot be termed strictly conformational in nature. This is in contrast to neutralizing mAbs produced against other enveloped viruses (Zwick et al., 2001, J. Virol. 75: 6692-6699; Wilson et al., 2000, Science 287: 1664-1666) that require the antigen to have native conformation for binding. It will be important to verify, under optimized conditions (Opstelten et al., 1995, J. Cell Biol. 131: 339-349) the use of viral lysates designed for maximal recovery of coronavirus proteins and to this end the production of high quality recombinant protein antigens will provide useful insights.

SARS-virus reactivity was confirmed for the four Western immunoblot negative, virus neutralizing mAbs (F26G3, G7, G9, G10) using an immunofluorescence assay. In order to independently confirm recognition of native SARS antigens we tested these mAbs via immunofluorescence relative to a non-neutralizing mAb F26G6, which we know recognizes Spike protein in immunohistochemical staining of infected Vero cells. The neutralizing mAbs F26G3, G7, G9, and G10 specifically recognize SARS-HCoV infected but not uninfected Vero cells in immunofluorescence (Fig. 2). Irrelevant, isotype matched mAbs, produced in an identical fashion, do not react with SARS-virus infected Vero cells. These data are consistent with the appearance of coronavirus antigens on the surface of the infected cell during replication (Talbot et al., 1984, Virology 132: 250-260) although the fixation process may allow for reactivity of these mAbs with internal antigens as well. Collectively, these data demonstrate that these mAbs will be useful for developing antigen detection systems for diagnostics.

Conclusions

Linear epitopes on the spike protein and conformational epitopes on as of yet unknown antigen(s) provide neutralizing targets on the SARS virus. These data clearly show that the spike protein is a putative protective antigen, as it is the target of neutralizing mAbs F26G18 and G19. Moreover, these mAbs could be used to identify protective epitopes for vaccine formulations (Enjuanes et al., 1995, Dev. Biol. Stand. 84: 145-152). Studies are underway to determine the identity of the additional unknown antigen(s) recognized by the other neutralizing mAbs with more conformational epitopes. Molecular studies have revealed that the RT PCR

amplified V-genes of the hybridoma clones that express these neutralizing mAbs contain distinct sequences. Therefore, the hybridomas expressing the neutralizing mAbs were derived from independently rearranged and clonally selected B cells in vivo, and are not derived from the same clone. This is the first description of SARS-HCoV specific and neutralizing mAbs and these antibodies should prove useful for the development of new diagnostic tests, studies on antigenic variation, and vaccine development in the global fight against SARS, as discussed above.

Virus, cells and monoclonal antibodies

Vero E6 (African Green Monkey kidney) cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) with 10% heat inactivated fetal bovine serum (FBS, Gibco BRL), 1% penicillin/streptomycin and 1% L-glutamine. Cells were incubated in the presence of 5% CO2 at 37°C.

The Tor3 strain of SARS CoV was isolated at the National Microbiology Laboratory from a patient infected during the initial SARS outbreak in Toronto 2003 (Weingartl et al., 2004, Emerg Infect Dis 10: 179-184). The virus stock had been expanded after plaque purification in Vero E6 cell monolayers and partially purified through a sucrose cushion (5 x 106 pfu/ml). Preparation of the infectious SARS CoV was performed under BSL-3 containment conditions. All animal experiments and processing of infected tissues were conducted under BSL4 containment conditions. Monoclonal antibodies were generated from mice immunized with inactivated SARS CoV Tor3 strain.

Animal Studies

Female BALB/c mice 6 to 8 weeks old were obtained from Charles River (Quebec, Canada). In the first mouse study, BALB/c mice were infected with the Tor3 strain of SARS CoV by one of three routes: intraperitoneal (IP), intranasal instillation (IN) or oral gavage (OR).

IN, IP and OR groups received 20 µl, 200 µl and 100 µl of diluted virus (containing 5x104 plaque forming units (PFU)) respectively, all animals received the same number of PFUs. At one hour and 1, 3, 5, 7 and 9 days post infection (p.i.), mice were anaesthetized with halothane and sacrificed by cardiac puncture. Blood, spleen, liver, kidney and lungs were harvested. Organs were immediately homogenized in DMEM immediately and an aliquot was removed for RNA

extraction. Remaining homogenates were stored at –80°C for virus isolation.

In a follow-up study, two groups of female BALB/c mice (6 to 8 weeks old, approximately 20 g in weight) were injected IP, a single time, with a cocktail of 4 neutralizing antibodies (Berry et al., 2004, J Virol Methods 120: 87-96). We administered 10 µg of each antibody to the mice; the final dose of antibody was therefore 40 µg/mouse. Two hours following antibody treatment, animals were anaesthetized with halothane and were inoculated IN with 5x105 PFU of the Tor3 strain in 100 µl. At 1, 2, 3, 4, 5, 6, 7 and 14 days following infection, mice from the antibody treated group (IN + AB) and untreated group (IN) were weighed then anaesthetized with halothane and sacrificed by cardiac puncture. Blood, spleen, liver, kidney, lung and brain were harvested. Organs were weighed then homogenized in 1.0 ml of DMEM, aliquots were transferred to AVL RNA extraction buffer (Qiagen) and stored at –20°C. The remainder of each homogenate was stored at –80°C for virus isolation. All animal experiments were performed under an approved animal use document and according to the guidelines of the Canadian Council on Animal Care.

RNA Extraction

RNA from the first animal experiment was extracted using the Trizol LS protocol (Invitrogen). RNA from the second animal study was extracted from tissue homogenates using Qiagen viral RNA Minikit (Qiagen). Homogenate was transferred to AVL extraction buffer and RNA was extracted following the Qiagen protocol.

Nested RT-PCR and Real-time RT-PCR

For the first mouse study, nested RT-PCR was performed using a primer set targeting the polymerase gene (L). RT-PCR was performed using a one-step RT-PCR kit (Qiagen) and primers CorV Forward1 and CorV 389 Reverse1 (Table 3) in a Biometra thermocycler. Nested PCR was done in a Biometra thermocycler using Taq DNA polymerase (Invitrogen) and primers

CorV 154 Forward2 and CorV 310 Reverse2 (Table 3) with 4% of the amplicons obtained from the first round reaction. All amplicons from first and second round amplifications were verified for size. All positive amplicons from the nested round were sequenced using an ABI 3100 Genetic Analyzer.

For the second mouse study, it was necessary to use real-time RT-PCR due to the large number of samples collected. RT-PCR master mixes were made using the Taqman one-step RT-PCR mastermix (Applied Biosystems) and primers targeting the nucleoprotein gene (Table 3) in an applied biosystems 7700 thermocycler.

Virus Isolation

Virus isolation was performed on selected tissue homogenates based on PCR data. Frozen homogenates were thawed from –80°C and centrifuged at 10,000xg for 5 minutes. Following centrifugation, supernatant was collected and mixed with 500 µl of DMEM (no supplements), and filtered using 0.22 µM filter (Millipore). Each supernatant was used to infect one 25 cm^2 flask of Vero E6 cells by incubation at 37°C for 1 hour with intermittent rocking. Five ml of DMEM containing 2% FBS, 1% penicillin/streptomycin and 1% L-glutamine was added to each flask. Cells were incubated at 37°C with 5% CO_2 and cytopathic effect (CPE) was monitored up to day 10 p.i. If CPE was present, supernatant was removed for testing in nested RT-PCR, followed by sequencing of amplicons.

Determination of viral load in the lung by TCID50

Tissue samples that demonstrated CPE upon first passage were chosen for TCID50 determination. Homogenized tissues in DMEM were filter sterilized using a 0.22 µM filter (Millipore) and diluted 1:100 in DMEM. Ten-fold serial dilutions from 10-2 to 10-8 were prepared in DMEM and used to infect Vero E6 cells at 80-90% confluency in 24-well plates. Media was removed from the cells and 250 µl of each dilution of virus was added to each of four wells. Virus was adsorbed to cells for 1 hour at 37°C, then 1 ml of DMEM with 2% FBS, 1% penicillin/streptomycin and L-glutamine was added per well. Infected cells were incubated at 37°C with 5% CO_2 and were monitored for CPE up to day 10 p.i. The dilution of virus that caused cytopathic effect (CPE) in 50% of the well was calculated by Spearman Karber method (Spearman, 1908, Brit J Psychol 2: 227). Virus titres are expressed as the 50% tissue culture infectious dose (TCID50) per gram of tissue.

Results

SARS CoV replication in mice infected by different routes

In order to establish a small animal model for efficacy testing of antivirals, vaccines and therapeutic antibodies, BALB/c mice were infected with the Tor3

strain of SARS CoV, $5x10^4$ PFU, by one of three routes: intraperitoneal (IP), intranasal (IN) or oral (OR). Animals were observed closely for clinical signs or symptoms over a period of nine days (1st study) and 14 days (2nd study) post virus challenge. Mice were serially sacrificed at different times p.i. and blood and organs were harvested for the detection of viral genomic RNA by RT-PCR and the presence of infectious virus by TCID50. In general, mice did not show any signs of disease, particularly not of respiratory illness. Intranasally infected animals demonstrated aggressive behaviour on days 3 and 4 p.i., however, no change in weight and grooming behaviour.

Independent of the route of infection, none of the animals were viremic at any time p.i. but virus spread systemically as indicated by replication in several organs, particularly spleen, liver and lungs (Figure 11). Of the three infection routes, the IP route was most efficient in initiating systemic infection more rapidly. Since the IP route does not mimic human SARS CoV transmission, of the routes that are biologically relevant for human transmission (IN and OR), IN infection was most successful with highest titres in spleen and lung. Despite the fact that OR infection did result in systemic infection, virus replication was only short lived compared to the IP and IN route. Viral RNA was not detected in any of the groups or tissues at day 9 p.i. indicating that the animals had cleared SARS CoV by that time. All RT-PCR positive amplicons were sequenced and confirmed to be SARS CoV.

Spleen and lung tissue samples from the biologically relevant routes (IN and oral) were selected for virus isolation to confirm the presence of viable virus in these tissues. Following infection of Vero E6 cells with tissue homogenates, CPE was observed on day 4. PCR amplification from RNA extracted from tissue culture supernatants followed by sequence determination confirmed the isolation of SARS CoV. Thus, we confirmed establishing a systemic infection with SARS CoV in mice by three different routes of inoculation. Infection by oral gavage is interesting since earlier reports suggest the possibility that SARS CoV can infect humans via the fecal/oral route (Tang et al., 2004, CMAJ 170: 47-54; Chan et al., Emerg Infect Dis 10: 825-831).

Neutralizing antibodies reduce virus titre

Having established a proper animal model with a relevant challenge route,

we next tested the neutralizing activity of several monoclonal antibodies raised against SARS CoV (Berry et al., 2004). We chose to use a 10-fold higher virus dose in a larger volume to infect the animals IN to assure a more reliable lower respiratory tract infection. Prior to IN infection of mice with SARS CoV (dose $5x10^5$ PFU), a cocktail of 4 neutralizing monoclonal antibodies (single dose) were administered IP. Animals were followed up by clinical observation and were sacrificed at different times post challenge. Tissue samples, collected post mortem were tested for the presence of viral nucleic acid by real-time RT-PCR and infectious virus by TCID50.

In accordance with the previous experiment, none of the infected animals demonstrated typical SARS illness. As demonstrated before, there was no detectable viremia, however there was systemic spread of infection, particularly to the spleen (day 2-6) and the lungs (day 1-14) in the untreated control group (Figure 12A). In comparison, the antibody treated group showed a dramatic decrease in viral replication in the spleen and lungs from day 3 on (Figure 12B). Viral replication was also observed in the brain on days 1 and 2 in the untreated group and only on day 1 in the antibody treated group.

To better define the neutralizing efficacy reduction in titre between the antibody treated (IN + Ab) and untreated groups (IN), titres were determined by TCID50 on lung homogenates. Mice that received the cocktail of neutralizing antibodies showed a two-log reduction in virus titre on day 1 and 3 p.i. (Figure 13). By day 4, the IN+AB group showed a reduction in titre by one-log in comparison to the IN-group. Furthermore, the viral load data was in concordance with the viral titre data and showed between one and three logs of decrease of viral RNA in the same samples.

Discussion

This study has demonstrated that SARS CoV established a systemic infection in mice following three different routes of virus infection without detectable levels of viremia. This is in contrast to the results of Subbaro et al., who recovered virus only from the upper and lower respiratory tract following intranasal infection but not from the internal organs (Subbarai et al., 2004, J Virol 78: 3572-3577). In our study, the main target organs for viral replication were determined to be spleen and lung and, thus, are similar to those in humans (To and Lo, 2004, J Pathol 203:

740-743; Wentworth et al., 2004, Emerg Infect Dis 10: 1293-1296). The virus replicated in the respiratory tract and spread systemically infected mice continued to gain weight and showed no signs of disease other than a marked increase is aggressive behaviour in IN infected mice on days 3 and 4 post infection viral RNA was detected in the brains of infected mice on days 1 and 2 post infection perhaps indicating limited infection via the olfactory bulb followed by inflammation on days 3 and 4 resulting in the observed aggression. We are confident that the mouse is a viable model for testing of antiviral, vaccines and immunotherapeutics as we are able to reliably induce systemic infection. However, as found by others groups protection can only be assessed by measuring reduction in virus replication (Subbarao et al., 2004), as mice are not a model for severe disease as none of the infected animals displayed typical SARS illness.

We attempted to determine if the SARS CoV could establish an infection in mice following oral inoculation. This was done in response to published data and our own observations indicating that viral RNA could be detected in human stool samples for up to 35 days, far longer than in the nasal swabs (Chan et al., 2004). In addition, the outbreak in Amoy Gardens, Hong Kong, appeared to be associated with fecal transmission raising the possibility of a fecal/oral transmission route for human SARS CoV infection (Ng, 2003, Lancet 362: 570-572; Department of Health, Hong Kong government. Outbreak of SARS at Amoy Gardens, available at http://www.info.gov.hk/info/ap/pdf/amoy_e.pdf). In our hands, the virus was clearly capable of initiating a systemic infection following oral infection with virus spread to the lungs, liver and spleen of the orally infected mice. We determined that infection via the intranasal (IN) route resulted in a more sustained and widespread respiratory and systemic infection than was observed following, either IP or oral infection and therefore, we chose to use this route for our subsequent work.

The current study demonstrated that IP administration of a single dose of a cocktail of neutralizing monoclonal antibodies prior to mucosal challenge reduced virus replication by two-logs in the first critical days p.i. . The antibody treated group showed a complete abolishment of viral RNA in all three tissues (spleen, liver, lung) 5 days after challenge while viral RNA was detected in the untreated group for up to 14 days in the lung. The ability of a single dose of neutralizing antibodies to inhibit virus replication in the lungs is promising since this is the

primary site of SARS replication and disease manifestation in humans. It is likely that consecutive treatments would enhance the efficacy particularly in humans at present his would be difficult to test experimentally as both NHP and mice are capable of clearing infection with the SARS-CoV independently of treatment and so we are limited to measuring viral loads early in the infection as an indicator of efficacy.

Previous studies have shown that infection as well as transfer of hyperimmune serum protects mice from IN challenge with SARS CoV (Subbarao et al., 2004). Although hyperimmune sera may work experimentally in mice, there are several problems associated with the use of polyclonal human sera in human patients such as difficulty in finding immune donors and risks related to the use of human blood products (Traggiai et al., 2004, Nat Med 10: 871-875). Recently, Traggiai and colleagues (Traggiai et al., 2004) demonstrated that human monoclonal antibodies offer an alternative. Mice were given between 50 and 800μg of human monoclonal antibodies IP and then challenged IN 2 days later with SARS CoV (104 TCID50). Animals that received 200μg of the human monoclonal antibodies were protected from viral replication in the lower respiratory tract, determined by TCID50 (Traggiai et al., 2004). However, RT-PCR detection was not employed to determine the levels of viral genome present in the tissues, typically a much more sensitive approach. Furthermore, only one time point was examined (2-days p.i.) and in our experience, even when using group sizes of 5 mice, it is possible that virus detection in the lung by RT-PCR or virus titration is negative at one time but positive later. We have shown that when administering the antibody cocktail containing a total of 40μg only 2 hours prior to challenge we can achieve a 2-log decrease in virus titre in the lung following infection with a 50x higher dose of SARS CoV ($5x10^5$ PFU). It is likely that possible the dose of antibodies, pre-treating earlier and/or multiple treatments to increase the tissue levels at the time of challenge will substantially improve the performance of the therapy. Furthermore, while a synergistic effect of these SARS-neutralizing monoclonal antibodies has not yet been demonstrated, the use of a cocktail of monoclonal antibodies should limit the potential deleterious effects of antigenic variation and escape from neutralization. Examples of synergistic effects of

monoclonal antibodies have been observed in the neutralization of HIV-1 in vitro (Zwick et al., 2001, J Virol 75: 12198-12208). Human monoclonal antibody therapy has also been studied in ferrets resulting in protection from SARS CoV challenge. However, at this time there appears to be little advantage in testing antibodies in this animal model (ter Meulen et al., 2004, Lancet 363: 2102-2103).

In conclusion, we have demonstrated for the first time that SARS CoV can cause systemic infection in mice when delivered by the IP, OR and IN routes. Despite the absence of any detectable viremia, viral RNA and infectious virus was primarily detected in lung and spleen. Furthermore, we have shown that administration of mouse monoclonal antibodies significantly reduces the viral load in primary target organs and protects animals from IN challenge. Thus, therapeutic antibodies have to be considered as a potential treatment option for SARS CoV infections in humans.

While the preferred embodiments of the invention have been described above, it will be recognized and understood that various modifications may be made therein, and the appended claims are intended to cover all such modifications which may fall within the spirit and scope of the invention.

Table 1: mAbs to the SARS HCoV Coronavirus

Clones	Class[1]	Neutralizing Titre[2]		Protein Target[4]	Conformational Requirement of Epitope in Immuno-dot blot[5]							IFA[6]	Epitope[7]
		NML	NCFAD[3]		N	H	D	HD	R	HR	A		
F26G1	G2a/k	0	0	Spike	+	+/-	+	-	+	+/-	-	+	L, E
F26G2	G2a/k	0	0	U	nd	nd	nd	nd	nd	nd	nd	-	C
F26G4	G2a/k	0	0	U	nd	nd	nd	nd	nd	nd	nd	—	C
F26G5	G2a/k	0	0	Spike	+	+	+/-	+/-	+	+	+/-	+/-	L, E
F26G6	G2b/k	0	0	Spike	+	+	+	+/-	+	+	+	++	L, E
F26G8	G2a/k	0	0	Spike	+	+	+	+/-	+	+	+/-	-+	L, E
F26G12	G2a/k	0	0	U	nd	nd	nd	nd	nd	nd	nd	-	C
F26G13	G2b/k	0	0	U	nd	nd	nd	nd	nd	nd	nd	+/-	C, E
F26G14	G2b/k	0	0	U	nd	nd	nd	nd	nd	nd	nd	+	C, E
F26G16	G1/k	0	0	U	+	-	+	-	-	-	-	-	C
F26G17	G2b/k	nd	0	U	nd	nd	nd	nd	nd	nd	nd	nd	C
F26G3	**G2a/k**	**>1/40**	**>1/20**	**U**	+	-	+	-	-	-	-	+	**C, E, P**
F26G7	**G2b/k**	**>1/80**	**>1/20**	**U**	+	-	+	-	+/-	-	-	+	**C, E, P**
F26G9	**G2a/k**	**>1/80**	**>1/20**	**U**	+	-	+/-	-	-	-	-	+	**C, E, P**
F26G10	**G2a/k**	**>1/80**	**>1/20**	**U**	+	-	+/-	-	-	-	-	++	**C, E, P**
F26G18	**G2b/k**	**nd**	**>1/20**	**Spike**	+	+/-	+	-	+	+	-	nd	**L, P**
F26G19	**G2a/k**	**nd**	**>1/20**	**Spike**	+	-	+	-	+/-	-	-	nd	**L, P**

[1] Only IgG class antibodies were used for this study.

[2] Virus neutralization tests were performed in independent containment laboratories (NML, National Microbiology Laboratory; NCFAD, National Centre for Foreign Animal Disease) laboratories independently.

[3] Only a single dilution of 1/20 was tested in microwell format.

[4] Protein specificity tests, shown here were determined by Western immunoblot with purified virus and infected cell lysate under denaturing conditions (Figure 1).

[5] Immunodot blot was performed using whole infected cell lysate separated into 6 different aliquots and then treated under various conditions described in methods. N, native ; H, heat denatured, 95°C for 5 minutes ; D, SDS treated (2%) ; H+D, heated in the presence of SDS (2%): R, treated with reducing agent, betarmercaptoethanol (5%) ; H+R, heated in the presence of reducing agent, betamercaptoethanol (5%); A, treated with heat, SDS (2%) and reducing agent

betamercaptoethanol (5%).

[6] Immunfluoresence on whole cell slides infected with SARS coronavirus (see Fig. 2) ; ++ strong positive reaction; + positive reaction; +/- weak positive reaction; - negative reaction.

[7] Epitope properties described as follows: L, linear or continuous epitope; E, surface exposed; C, conformational epitope ; P, protective epitope in vitro; nd, not determined; neutralizing clones are embolded; U, Unknown

Table 2

Bio-Activity	mAb	Western Reactivity	ELISA REACTIVITY Viral Lysate[a]	Denatured Lysate[b]	O.D. Reduction Fold[c]	Percent	Mean[d]
non-neutralizing	F26G2	-	0.743	0.424	1.7	43	51
	F26G4	-	0.751	0.363	2.1	52	
	F26G5	-	1.224	0.383	3.2	69	
	F26G12	-	0.533	0.338	2.9	37	
	F26G13	-	1.048	0.481	2.2	54	
	F26G14	-	1.448	0.633	2.3	56	
	F26G16	-	2.037	1.534	1.3	25	
	F26G17	-	1.986	0.560	3.5	73	
	F26G1	+	1.709	0.584	2.9	66	
	F26G6	+	1.600	0.600	2.7	62	
	F26G8	+	1.408	0.497	2.8	29	
	F26G15	+	1.134	0.604	1.9	47	
neutralizing	F26G3	-	1.253	0.276	4.5	78	78*
	F26G7	-	1.917	0.382	5.0	80	
	F26G9	-	1.345	0.278	4.8	79	
	F26G10	-	1.259	0.290	4.3	77	
	F26G18	+	1.807	0.501	3.6	72	
	F26G19	+	1.505	0.253	6.0	83	

[a]Native gradient purified virus coated at 32 ng/well total protein
[b]Denatured Virus was also coated at 32 ng/well after heating at 100°C for 10 minutes.
[c]Fold reduction in OD at 405nm
[d]Mean calculated based on groups of non-neutralizing or neutralizing monoclonal antibodies
*p<0.001, students T-Test

This table depicts further ELISA characterisation of the nature of the epitopes. The neutralizing mAbs in general have a higher dependence on integrity of the native structure for binding.

Table 3 Oligonucleotides used to amplify SARS CoV viral RNA

Primer Name	Target Gene	Purpose	Sequence 5' to 3'	Size of Amplicon
CorV 1 Forward	pol	RT-PCR	cagagccatgcctaacatg	389 bp
CorV 389 Reverse1	pol	RT-PCR	aatgtttacgcaggtaagcg	
CorV 154 Forward2	pol	Nested PCR	tgttaaaccaggtggaac	310 bp
CorV 310 Reverse2	pol	Nested PCR	cctgtgttgtagattgcg	
Forward Primer	np	Real-time PCR	accagaatggaggacgcaatg	NA
Reverse Primer	np	Real-time PCR	gctgtgaaccaagacgcagtattat	
TaqMan MGB probe	np	Real-time PCR	(FAM)-accccaaggtttaccc	NA

FAM is 6-carboxyfluorescein reporter dye